1 Experimental Design and Data Collection

Maria Rasch

CONTENTS

1.1 EXPERIMENTAL DESIGN

Assessments of the impact of environmental factors on the response of food-borne microorganisms are the primary sources of data for the development of predictive models. When investigating the influence of more than one factor and accurately describing how those factors interact, it is important to consider how to design the experiment. Unfortunately, in modeling bacterial growth in foods, the design is commonly not accounted for, or it is chosen based on habit rather than the experiment's specific purpose. But carefully considering the experiment's design is vital to extracting the desired information (e.g., interactions) and to avoiding excessive experimental work. Furthermore, researchers should be aware of the experimental design in order to avoid extrapolation.[1–3] The following sections describe the most common experimental designs used in modeling of microbial responses in food.

1.1.1 Complete Factorial Design

A complete factorial design is one in which all combinations of the different factors are investigated (Figure 1.1). This allows straightforward modeling of interactions between, for example, environmental factors influencing growth or inactivation of microorganisms. The experimental design is simple, easy to set up, and easy to handle statistically. The main disadvantage is the large increase in number of experiments for every new factor/level added to the experiment. A simple example of a complete $3 \times 3 \times 3$ factorial design was applied by Chhabra et al.[4] for investigating thermal inactivation of *Listeria monocytogenes* in milk. The factors were milk fat content, pH, and heating temperature; the experiment was performed in triplicate, resulting in $3^4 = 81$ experiments. A complete factorial experimental design was also used by Uljas et al.[5] for modeling the combined effect of different processing steps on the reduction of *Escherichia coli* O157:H7 in apple cider. The response variable measured was binary (whether a 5-$\log_{10}$-unit reduction was obtained or not), resulting in a logistic model. Three class variables (cider from three different cider plants, a freeze–thaw treatment, and the preservation agents potassium sorbate and sodium benzoate) and four continuous variables (cider pH, storage temperature, storage time, and preservation concentration) were investigated.[5] This resulted in 1,596 treatments for each of the three types of cider. As one type of cider was tested in duplicate and the other two in triplicate, the total number of experiments was 12,768, which very clearly illustrates the major drawback of complete factorial designs, namely, the very large number of experiments required. However, complete factorial designs are still widely used within predictive modeling of microorganisms, and have been used for different purposes such as the effect of inoculum size, pH, and NaCl on the time-to-detection (TTD) of *Clostridium botulinum*;[6] the effect of pH, NaCl, and temperature on coculture growth of *L. monocytogenes* and *Pseudomonas fluorescens*;[7] the effect of temperature, NaCl, and pH on the inhibitory effect of the antimicrobial compound reuterin on *E. coli;*[8] and the growth of *L. monocytogenes* under combined chilling processes.[9]

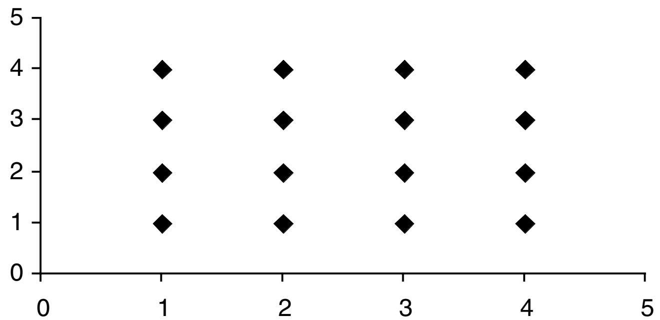


FIGURE 1.1 Example of a complete factorial design for the simple case with two variables ($k = 2$), e.g., temperature and pH, each at four levels.

1.1.2 Fractional Factorial Design

In order to reduce the number of experiments, several different alternative experimental designs can be applied. Among these are fractional factorial designs, described in this section. In contrast to the complete factorial designs, the fractional factorial designs are not as easy to construct, and thus different software packages are often used for determining which combinations of the different parameters to include in the experimental setup. Examples of software programs used for fractional factorial designs are the Screening Design procedure from STATGRAPHICS Plus (Manugistics, Rockport, MD)[10] and Modde (Umetri, Umeå, Sweden).[11] Farber et al.[10] used a fractional factorial design for modeling the growth of *L. monocytogenes* on liver pâté. The factors investigated were temperature, salt, nitrite, erythrobate, and spice, each at two different levels. The fractional factorial design yielded a total of 16 experiments (= 2^{5-1}), where a complete factorial design would have resulted in 32 different experiments. Juneja and Eblen[12] also obtained a large reduction in number of experiments (compared to the number of experiments in a complete factorial design) when they modeled thermal inactivation of *L. monocytogenes.* They investigated 47 combinations of four different environmental factors (temperature, NaCl, sodium pyrophosphate, and pH, each at five levels), where a complete factorial design would have resulted in $5^4 = 625$ experiments. Fractional factorial designs have also been applied for investigating the heat resistance of *E. coli* O157:H7 in beef gravy.[13]

A particular class of fractional factorial designs has been widely used for modeling of bacterial growth, namely, the Box–Behnken designs. These designs are formed by combining two-level factorial designs with balanced incomplete block designs (Figure 1.2).[14] Often, more than one experiment is performed at the central point of the experimental design in order to evaluate the repeatability of the model. A Box–Behnken design was applied for three studies of spoilage of cold-filled ready-to-drink beverages investigating the bacteria *Acinetobacter calcoaceticus* and *Gluconobacter oxydans,*[15] the molds *Aspergillus niger* and *Penicillium spinulosum*,[16] and the yeasts *Saccharomyces cerevisiae, Zygosaccharomyces bailii,* and *Candida*

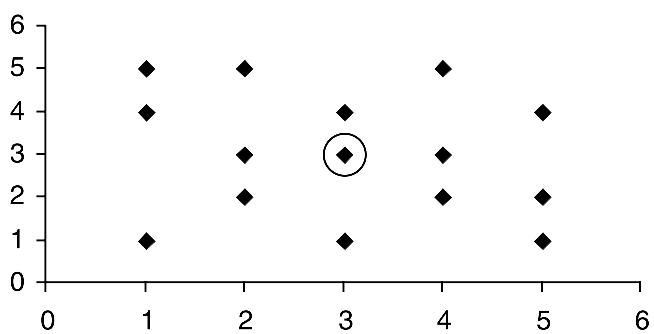


FIGURE 1.2 Example of a Box–Behnken design for the simple case with two variables ($k = 2$), e.g., temperature and pH. The circle denotes the central point.

lipolytica.[17] In each study, the effect of pH, titratable acidity, sugar content, and concentrations of the preservatives sodium benzoate and potassium sorbate were tested at three different levels each.[15–17] The Box–Behnken design was constructed by using the JMP software (SAS Institute, Cary, NC), with two points at the center of the design, resulting in 42 experiments.[15–17] A Box–Behnken design has also been used to show that the CO_2 concentration in the water phase of a model food system was the most important factor when describing modified atmosphere packaging and its inhibitory effect towards microorganisms.[18] The CO_2 concentration in the water phase was investigated as a function of gas/product ratio, initial CO_2 concentration in the gas phase, temperature, pH, and lard content.[18]

1.1.3 Central Composite Design

A central composite design consists of a complete (or fraction of a) 2^k factorial design, n_0 center points, and two axial points on the axis of each design variable at a distance of α from the design center (Figure 1.3). The number of experiments for k variables is $2^k + 2k + n_0$, where n_0 denotes the number of experiments at the central point ($n_0 \geq 1$).[14] For $k = 2$ and 3 and $n_0 = 2$, this results in 9 and 16 experiments, respectively.

In a validation study by Walls and Scott,[19] the effect of temperature, pH, and NaCl on the growth of *L. monocytogenes* was described by the use of a central composite design. The experiment was repeated six times at the design center in order to estimate the experimental variance. Guerzoni et al.[20] used central composite design to optimize the composition of an egg-based product in order to prevent survival and growth of *Salmonella enteritidis*. The factors studied were pH, NaCl, and pressure treatment. Lebert et al.[21] used a central composite design to study the growth of *L. monocytogenes* in meat broth. Three variables were studied: pH, a_w,

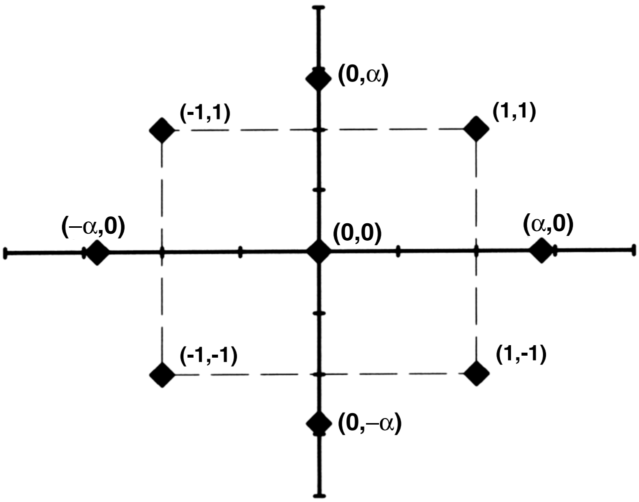


FIGURE 1.3 Example of a central composite design for the simple case with two variables ($k = 2$), e.g., temperature and pH.

and temperature. Two experiments were performed at the central point, resulting in 16 experiments. Later, a similar design was used to study the mixed growth of *Pseudomonas* spp. and *Listeria* in meat, where the three variables were NaCl, temperature, and pH.[22]

A combination of two central composite designs and a factorial design has been applied to study the effect of osmotic and acid/alkaline stresses on *L. monocytogenes.* The two central composite designs were set up in the acid and alkaline pH range, i.e., one covered the pH range from 5.6 to 7 and the other from 7 to 9.5.[23,24] As pointed out by Pin et al.,[2] the risk of extrapolation can be very high when using central composite design as the vertices of the nominal variable space (the unit cube) are far from the interpolation region (the minimal convex polyhedron). The shape of the minimal convex polyhedron is determined by a convex linear combination of the environmental factors at which the experiments were performed for the model development. If a prediction is made randomly in the unit cube, the risk of extrapolation is as high as 75%.[2]

1.1.4 Doehlert Matrix

The Doehlert matrix is another form of experimental design that to some extent resembles the central composite design. The Doehlert matrices consist of points uniformly spaced on concentric spherical shells, and are therefore also called uniform shell designs (Figure 1.4).[14] The number of experiments for k variables is $k^2 + k + n_0$, i.e., for $n_0 = 1$ this gives 13 experiments for $k = 3$ and 21 experiments for $k = 4$. The experiment performed at the center of the experimental domain (n_0) can be repeated several times in order to estimate residual variance. An advantage of

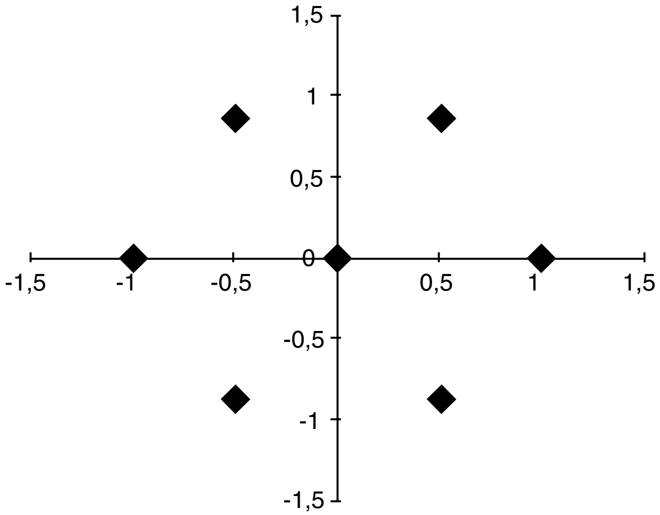


FIGURE 1.4 Example of a Doehlert matrix design for the simple case with two variables ($k = 2$), e.g., temperature and pH.

Doehlert matrices is that they are easy to expand. Expansion can be done both by investigating new variables (provided that these variables were set to their central point during the first experiments) and by enlarging the range of the parameters tested without having to repeat the former experiments.[25]

The Doehlert matrix has been used by Terebiznik et al.[26] to investigate the effect of combinations of nisin and pulsed electric fields on the inactivation of *E. coli*. The experiments were designed with three variables, namely, nisin concentration, electric field strength, and number of pulses. In a later study by the same group, the effect of water activity in combination with nisin and electric field strength was studied.[27] Bouttefroy et al.[28,29] used Doehlert design to study the inhibition of *L. monocytogenes* at different combinations of NaCl, pH, incubation time, and the inhibitory effect of the bacteriocins nisin and curvaticin 13, respectively. Doehlert design has also been applied to investigate the conidial germination of *Penicillium chrysogenum* at different combinations of temperature, water activity, and pH.[30]

1.1.5 Optimal Experimental Design

A new approach within mathematical modeling of growth or inactivation of microorganisms is optimal experimental design. The basic idea is to optimize the experimental conditions with respect to parameter estimation by the use of an established methodology from bioreactor engineering.[31] Ideally, the optimal design of dynamic experiments will result in increased information content from each experiment, and thereby to more accurate parameter estimates from a smaller number of experiments. In the approach by Bernaerts et al.,[32,33] the growth data are modeled directly by the square root model of Ratkowsky et al.[34] (see Chapter 3) integrated into the dynamic model of Baranyi and Roberts[35] (see Chapter 2). Thus, the so-called secondary model parameters are estimated directly from the population density data. Optimal dynamic experimental conditions are then obtained by a stepwise change in temperature, which is first shown with a one-step change[32] and later with three smaller temperature increments in order to avoid an intermediate lag phase.[33] The optimization process was performed by designing the optimal step-temperature profile in order to minimize the standard deviation of the parameter estimates and the correlation between parameters.[32,33] Grijspeerdt and Vanrolleghem[36] used optimal experimental design for optimizing sampling times for the Baranyi growth model. This resulted in lower error on the parameter estimates and decreased correlation between them.[36]

1.2 DATA COLLECTION

1.2.1 Strain Selection

There are several different approaches that one can use when choosing which strain to use for model building purposes. Furthermore, there is the choice between using a single strain or a mixture of different strains (i.e., cocktail). Before choosing which strain to use it is important to clarify the intended use of the model: is the model going to be used for prediction of possible growth of one particular pathogenic species, or is it a model of the spoilage flora of a specific food product? Using a strain (type strain

or other) that has previously been used for several studies or maybe even modeling purposes gives the benefit of the previously accumulated knowledge on the particular strain. On the other hand, a strain isolated from the particular food product which is the goal for the application of the model gives the advantage of relevance for the product, and being able to grow the strain at the environmental conditions investigated.

A well-known strategy is to choose the fastest growing strain at the environmental conditions investigated, as it is the fastest growing strain that will dominate the growth in, e.g., food products. McMeekin et al.[37] recommended independent modeling of several different strains before choosing the strain that grows fastest under the environmental conditions of most interest. This strain then simulates a worst-case scenario.[37] This strategy was followed by Neumeyer et al.[38] who, after an initial screening of different *Pseudomonas* strains, chose the fastest growing strain for modeling, and later during the validation stage confirmed that the chosen strain was the fastest strain.[39] For modeling the growth of *Bacillus cereus* in boiled rice, three different strains were examined and the fastest growing of the three chosen for the modeling studies.[40] Miles et al.[41] examined four different strains of *Vibrio parahaemolyticus* and found that one strain was the most resistant at all conditions of temperature and water activity tested, and hence the growth data of this strain were used for model development. A different method was employed by Lebert et al.[22] who modeled the growth of three different strains, one fast and one slow growing strain of *Pseudomonas fragi* and one slow growing strain of *P. fluorescens*. Any growth was then assumed to be within a zone delimited by the predicted growth curves of these three different organisms.[22] A similar approach was followed by Benito et al.,[42] who initially investigated the resistance of six different strains to high hydrostatic pressure and heat before choosing one pressure- and heat-resistant strain and one pressure- and heat-sensitive strain for further analyses.

The strains used for model development can also be isolated from the food that is under investigation. For modeling the spoilage of ready-to-drink beverages, strains of *Saccharomyces cerevisiae*, *Z. bailii,* and *C. lipolytica* were used.[17] These strains were all isolated from spoiled ready-to-drink beverages.[17] Oscar[43] chose a specific strain of *Salmonella typhimurium* as it exhibits the same growth kinetics as *Salmonella* strains commonly found on chicken in the U.S. The use of strains related to the food in question was also recommended by Hudson,[44] who used strains isolated from smoked mussels and sliced smoked salmon to investigate the growth of *L. monocytogenes.*

The importance of using more than one strain of a species in order to assess the influence of strain variation has also been stressed.[44–46] According to Whiting and Golden,[46] the between-strain variation should be equal to or smaller than the experimental and statistical variation. However, when investigating the growth, survival, thermal inactivation, and toxin production by 17 different strains of *E. coli*, they found that the variations among the strains were larger than the uncertainties related to the experimental error.[46] The variation among 58 strains of *L. monocytogenes* and 8 strains of *Listeria innocua* was examined by Begot et al.[45] Most of the strains had been isolated from meat, meat products, and related industrial sites, and four additional strains that had been involved in outbreaks were also included. Large variations in lag times were found between the strains, whereas the variations in generation

times were less pronounced.[45] The opposite conclusion was reached by Oscar[47] when studying 11 different strains of *Salmonella*. He found that the mean coefficients of variation for four repetitions with the same strain were 11.7 and 6.7% for the lag time (λ) and the specific growth rate (μ), respectively, whereas the mean coefficients of variation among the different strains were 9.4 and 5.7% for λ and μ, respectively.[47]

Salter et al.[48] compared the growth of the nonpathogenic *E. coli* M23 with the growth of different pathogenic strains of *E. coli* and found only little difference in the growth responses of the different strains. They also found that the model based on *E. coli* M23 was able to describe the growth of pathogenic strains of *E. coli*, including *E. coli* O157:H7.[48] This result has practical value, as many research groups do not have access to laboratory facilities suitable for work with *E. coli* O157:H7. However, the general suitability of nonpathogenic strains as models for the growth or survival of pathogenic strains would have to be confirmed for each species.

Mixtures of different strains, so-called cocktails, have also been widely used. The main arguments for using cocktails are as follows: first, that a mixture of several different strains is more representative of the situation found in foods, where a flora of strains is likely to be present. Second, it is not necessarily the same strain that shows the fastest growth under all the investigated growth conditions, i.e., a strain with a high salt tolerance might be the fastest growing at high salt concentrations and high pH, but not necessarily at low salt and low pH conditions. For building the Food Micromodel, which is a database software system for predicting growth and survival of microorganisms in foods (see Chapter 6), it was decided to use a cocktail of strains for the growth experiments, but a single strain for thermal inactivation studies, as a cocktail of strains for the latter procedure could produce thermal inactivation kinetics data that would be difficult to interpret.[49]

A cocktail of five strains of *Staphylococcus aureus* was used for the determination of growth/no growth boundaries by measuring turbidity in microtiter plates[50] and Uljas et al.[5] used a mixture of three different strains to characterize the effect of different preservation methods on the survival of *E. coli* in apple cider.

1.2.2 Viable Count

Viable count determinations by spreading on agar plates are still a very common method for enumeration of microorganisms and it remains the method of reference. To a certain extent it has been possible to automate viable count plating by the use of automated platers such as the spiral plater and automatic colony readers.

Vast numbers of modeling studies have been based on viable counts. A few studies have, however, observed problems with the viable count method compared with other methods. As described in Section 1.2.3.2, enumeration of *Brochothrix thermosphacta* by flow cytometry gave a more accurate result than with viable counts when both were compared to manual counting by microscopy.[51]

1.2.3 Novel Methods

Construction of models using viable count data is time-consuming and expensive, and several alternative, more rapid methods for accumulating sufficient data for

modeling have been explored. A novel method for data capture should either be faster, cheaper, and less labor intensive or be able to provide more information on the cells than do viable counts, e.g., physiological status or expression of different phenotypic traits. In the following sections, four of these novel methods will be described, namely, turbidity, flow cytometry, microscopy, and impedance. A number of other methods have been used to indirectly model bacterial growth, but extensive development of these approaches has not been attempted. Some of these include headspace measurements of evolved CO_2 by gas chromatography[52,53] and bioluminescence.[54,55]

1.2.3.1 Turbidity

One of the simplest methods for data collection is the use of optical density (OD), where growth can be related to the increase in turbidity of a bacterial culture. OD, or absorbance, is a measure of the amount of light that is absorbed or scattered by a solution of bacteria. The bacteria absorb or scatter light depending on their concentration, size, and shape. According to Beer's law, absorbance is proportional to concentration, and is related to the percent transmitted light (*%T*) by the following equation:

$$OD = 2 - \log_{10}(\%T)$$

Some of the fundamentals of this approach have been discussed by McMeekin et al.[37] There are some limitations associated with this approach to data collection. Deviations from responses predicted by Beer's law occur at high cell densities, requiring that dilutions be made to OD < 0.3 before accurate absorbance measurements can be taken.[56] In addition, OD methods are comparative only, and cannot be used to predict viable counts unless some attempt at calibration is made. Detectable absorbance changes occur at a minimum bacterial concentration of 10^6 cfu ml^{-1}, depending on the sensitivity of the instrument,[56] and a linear relationship between OD and viable count exists only between the detection limit and approximately $10^{7.5}$ cfu ml^{-1}. With the maximum cell density in most growth media limited to approximately 10^9 cfu ml^{-1}, the μ measured using OD will represent the rate towards the end of the growth phase, and this will be less than the maximum specific growth rate (μ_{max}) experienced during the midexponential phase of growth. Another drawback is the inability to distinguish between dead and living cells, which can lead to an overestimation of the cell concentration. Furthermore, bacterial cultures that change cell morphology under different environmental conditions, e.g., elongated cells of *L. monocytogenes* at high salt concentrations, again lead to an overestimation of the cell number.[57] Hudson and Mott[58] showed that the cell length of *P. fragi* increased during lag phase, and consequently models based on OD measurements underestimated λ, unless a conversion equation was applied.[58] This method lends itself particularly well to automation, and a number of studies have used automated turbidimetric instruments such as the Bioscreen.[57,59,60]

A number of attempts have been made to calibrate OD data. McClure et al.[57] used a simple quadratic equation to relate OD to viable counts. Dalgaard et al.[56]

used two equivalent methods for calibration: one in which stationary-phase cells were diluted to the appropriate OD, and the other in which samples for OD and viable count were taken during growth. Predicted generation times were lower with viable count data,[56] and this factor has been taken into account in later studies.[41] Similar methods have been used to relate turbidimetric and viable count data.[58,61,62]

In some studies, the Gompertz equation was fitted directly to OD data; however, no data were available at below the minimum detectable OD (ca. 10^6 cfu ml^{-1}) and thus the estimates for μ and μ_{max} should be questioned.[44,58] A form of calibration was achieved by relating λ determined using OD measurements to that determined with viable counts by a regression equation.[58] McMeekin et al. have discussed the correct way to fit the Gompertz function to % transmittance data (Appendix 2A.9 of their book[37]), and this method has been used to calculate generation times.[38]

Other studies have been carried out without any apparent calibration.[59] λ values have been estimated from OD data by extrapolation of the exponential portion of the curve back to the initial cell numbers;[63] however, this method may be inaccurate since the μ estimated from the OD data may be lower than that obtained during the period of maximum growth.[37] Lebert et al.[21] estimated λ values of *L. monocytogenes* with OD data, but the inoculation level during these experiments was kept at 10^7 cfu ml^{-1}, i.e., above the detection limit. This procedure, however, gives only a very small dynamic range of growth of about 2 log units.

Interestingly, the TTD approach has not been used to any great extent. The TTD for a turbidimetric instrument can be defined as the time required for a detectable increase in OD. The difference between TTD for serial twofold dilutions gives the doubling time, from which μ can be determined.[60,64] λ can be calculated subsequently by the difference between the predicted TTD based on λ, and the observed TTD.[60,64] This method was used to estimate λ for individual cells.[65] This method was also used by Augustin et al.[61] for estimating μ_{max} of 10 different strains of *L. monocytogenes*. They, however, observed large variations in the time separating the two successive growth curves (i.e., doubling time).

In spite of the problems associated with the use of turbidimetric data for modeling, there appears to be some value in this approach. Models based on viable counts were compared with those obtained using either OD or transmittance data, and it was concluded that turbidimetric methods may be used for reliably estimating μ_{max}.[56]

OD measurements have been used extensively for modeling purposes. This includes modeling of the growth boundaries of *S. aureus* at different levels of relative humidity, pH, potassium sorbate, and calcium propionate[50] and modeling the effect of the antimicrobial compound reuterin on the growth of *E. coli* at different combinations of temperature, pH, and NaCl.[8] OD has also been used to determine 5-log_{10}-unit reductions of *E. coli* in apple cider (see also Section 1.1.1).[5] Cider inoculated with 10^7 cfu ml^{-1} was exposed to the different treatments investigated, after which a 10-μl sample of the cider was transferred to a microtiter well containing Tryptic Soy Broth and incubated. If a 5-log_{10}-unit reduction occurred during the treatment, the 10-μl sample would contain <1 cfu and therefore no growth would be observed in the well.[5]

OD data have also been used for the determination of growth boundaries, i.e., the growth/no growth models (see Chapter 3). The growth boundaries of the spoilage

organism *Z. bailii* were investigated at different combinations of salt, sugar, acetic acid, and pH at a constant temperature of 30°C. Growth was measured by a Bioscreen analyzer, but between measurements the Bioscreen plates were incubated in closed containers in an incubator.[66] Masana and Baranyi[67] studied the growth boundaries of *B. thermosphacta* in multi-well plates, but inspected the wells visually for growth. The interface between survival and death of *E. coli* O157:H7 in a mayonnaise model system was studied by McKellar et al.[68] A cocktail of five strains of *E. coli* O157:H7 was inoculated at a level of 10^7 cfu ml^{-1} into 5-ml tubes under different environmental conditions, and growth was observed visually. In the case of no growth, the samples were diluted 100-fold into Tryptic Soy Broth and incubated again. Continued absence of growth was interpreted as a >5.7 log reduction in viable cell numbers under the test conditions. Survival was hence defined as a <5.7 log reduction in viable cell numbers.[68]

1.2.3.2 Flow Cytometry

Flow cytometry is a rapid technique for measurement of single cells in suspension. Individual cells confined within a rapidly flowing jet of water pass a measuring window, in which several parameters can be simultaneously measured for several thousand cells per second with high precision.[69] Light scattering reflects cellular size and structure, while fluorescence measurements can determine the cellular content of any constituent that can be labeled with a fluorescent dye.[70] In this way flow cytometry combines the advantages of being a single cell technique with the power of being able to measure a very large number of cells in a very short time. The resulting data are not a mere average of the measured cells but a distribution of the measured parameters for the cells. The possibility of measuring the distribution gives an estimate of the heterogeneity of the microbial population and thereby also the possibility to detect subpopulations that, e.g., are resistant to a treatment under investigation. With a flow cytometer equipped with a cell sorter it is furthermore possible to sort cells out on the basis of the parameters measured. These cells can then be sorted into microtiter wells and be used for new growth experiments to monitor, e.g., λ for the single cells as shown by Smelt et al.[71] In general a good correlation between the number of cells determined by plate counting and by flow cytometry has been found for both bacteria[72] and yeast,[73] with detection limits of approximately 10^4 and 10^2 cells ml^{-1} determined for *L. monocytogenes* and *Debaryomyces hansenii*, respectively.

The use of flow cytometry for predictive microbiology is still very limited. Sørensen and Jakobsen[73] used flow cytometry to enumerate viable cells of *D. hansenii* at different environmental conditions. The growth data were used to model λ and μ_{max} as a function of temperature, pH, and NaCl. Rattanasomboon et al.[51] compared flow cytometry, turbidimetry, plate counts, and manual counts by microscopy for enumeration of *B. thermosphacta*. They found that turbidimetry overestimated the cell number as the *B. thermosphacta* cells changed morphology during growth, whereas flow cytometry gave a more accurate cell count than did plate counts when both were compared to manual counts.[51] This overestimation of cell number and hence μ could not be confirmed by Dalgaard and Koutsoumanis,[74] who found that turbidimetric measurements estimated μ_{max} and λ accurately.

Possible applications of flow cytometry include enumeration of microorganisms for both mono cultures and mixed cultures,[72,75] direct measurement of lag phase as described by Ueckert et al.,[76] separation of intermediate states between dead and culturable cells,[77] and detection of cell injury caused, e.g., by bacteriocins.[78]

However, flow cytometry also has the potential to be used for gaining more information on the microorganisms than just the number of cells. García-Ochoa et al.[79] recognized that in order to develop a structure kinetic model for the production of xanthan by *Xanthomonas capestris*, they needed quantitative data on intracellular compounds. They examined the DNA, RNA, and intracellular protein content by flow cytometry and traditional biochemical methods, enabling them to set up standard curves and thereby quantify these intracellular compounds by flow cytometry. It is also possible to determine other biochemical parameters such as intracellular esterase, protease, glycosidase, and phosphatase activities. One of the limitations in the use of flow cytometry is that it can be applied for liquid systems only. This problem was, however, partly overcome by de Alteriis et al.,[80] who studied the growth dynamics of *Saccharomyces cerevisiae* cells immobilized in a gelatin gel. When the cells were sampled for analysis, the gelatin was enzymatically liquefied with trypsin, thus enabling the cells to be analyzed by flow cytometry.

1.2.3.3 Microscopy and Colony Size

Microscopy is another method that is gaining interest as developments in image analysis programs and software tools for automation make the method more feasible. Microscopy enables direct studies of single cells, which give new opportunities for following the same cells for longer periods of time. One of the main advantages of microscopy and the measurement of colony size is the possibility of studying solid systems, which more closely resemble the situation in most food systems. It is, however, also possible to investigate growth in a liquid system. By the use of a microscope coverslip coated with, e.g., poly-L-lysine, it is possible to obtain immobilized cells in a liquid system, as has been demonstrated for both yeast[81] and bacteria.[82]

Reports on the use of microscopy for predictive modeling of single cells are still sparse. Wu et al.[83] recently compared the use of microscopy for determination of lag phase duration for individual cells of *L. monocytogenes* with the TTD method (described in Section 1.2.3.1). Microscopy has several advantages over the TTD method for the determination of λ of single cells. The method is a direct method allowing visual observation of the first cell division, whereas the TTD method depends on the time of detection, the growth rate, and extrapolation back to the single cell. Furthermore, any treatment that results in cells not dividing will not be detected by the TTD method.[83] A drawback when studying single cells by microscopy can be the difficulties in obtaining sufficient data for modeling purposes. Wright et al.[84] used a gel-cassette in which bacteria grow as colonies immobilized in gelatin gel, combined with a "laser gel-cassette scanner," to study the lag and doubling time of *Salmonella typhimurium* at different concentrations of NaCl and pH. The inoculated gel-cassette was continuously scanned, and the increase in fixed angle laser light scattering intensity was related to the increase in diameter of the individual

nearly spherical bacterial colonies within the controlled environment of the gel-cassette. The system, however, needs extensive calibration; for example, it is necessary to recalibrate for each new experiment in order to relate laser scattering intensity to viable cell count.[84]

Radial growth of *L. monocytogenes* and *Yersinia enterocolitica* was studied on agar surfaces under different modified atmosphere conditions.[85] Growth of visible colonies was followed by image analysis and viable count per colony. A linear relationship was found between $\log_{10}$ viable cell number per colony and $\log_{10}$ colony radius and μ.[85] Dykes[86] used a similar method to investigate sublethal injury in *L. monocytogenes.* Cells subjected to either starvation or heat stress were plated onto Tryptic Soy Agar and incubated at 37°C for 48 h. The plates were photographed using a digital camera and the areas (mm^2) of individual colonies were determined using image analysis. The results were presented as histograms showing frequency distribution of colony area. The colony areas from nonstressed cells were normally distributed, whereas the colony areas from starved or heat-stressed cells had a skewed distribution due to an increased proportion of small colonies.[86] The growth of *Bacillus cereus* was also measured as radial growth at different concentrations of agar, NaCl, and potassium sorbate.[87] Agar plates were incubated at 30°C and photographs were taken at 30-min intervals. The colony diameters were measured on the slides, and the time to reach a diameter of at least 0.1 mm was called "time to visible growth." Growth was then evaluated as time to visible growth or radial growth rate.[87] Time to visible growth was also measured by Salvesen and Vadstein[88], although they defined a colony as visible when it reached a diameter of 2 mm. They studied seawater isolates and found an inverse relationship between the μ_{max} determined in liquid culture and the time necessary to form visible colonies on agar.[88]

In contrast to bacteria, the growth of molds is usually always measured as radial growth since molds are not unicellular. Gibson et al.[89] first modeled μ and the time to visible growth (diameter ≥ 3 mm) for fungi, where the growth of *Aspergillus flavus* was modeled at different water activities. Valík et al.[90] also modeled the effect of water activity but on *Penicillium roqueforti*. The diameter of the colonies was fitted to the model of Baranyi et al.[91] (see Chapter 2), and λ and μ modeled as a function of water activity. Later Valík and Piecková[92] used the same approach to model the effect of water activity on three different heat-resistant fungi, namely, *Byssochlamys fulva*, *Neosartorya fischeri,* and *Talaromyces avellaneus.* Recently, Rosso and Robinson[93] proposed a model to describe the effect of water activity on the radial growth of molds. The model is of the cardinal model family (see Chapter 3) and fitted successfully the radial μ of six different *Aspergillus* species as well as *Eurotium amstelodami*, *Eurotium chevalieri,* and *Xeromyces bisporus.*

1.2.3.4 Impedance

Microbiological impedance devices measure microbial metabolism in medium by tracking the movement of ions between two electrodes (conductance), or the storage of charge at the electrode–medium interface (capacitance). For bacterial growth, the conductivity of the growth medium increases with bacterial numbers because of the production of weakly charged organic molecules.[37] This production of charged

molecules is due to, for example, the conversion of proteins to amino acids, carbohydrates to lactate, and lipids to acetate, all of which will increase the conductivity (*G*) of the growth medium.[94] When electrodes are immersed in a conductive medium, a dielectric field will build up at the electrode–solution interface. The medium will display a capacitance due to the polarization of the electrode–solution interface. An alternating sinusoidal potential applied to the system will therefore cause a resultant current depending on the impedance (*Z*) of the system, which is a function of its resistance (R, $G = 1/R$), its capacitance (*C*), and the applied frequency (*f*).[94]

$$Z = \sqrt{\left(\frac{1}{G}\right)^2 + \left(\frac{1}{2\pi fC}\right)^2}$$

Which signal should be measured (impedance, conductivity, or capacitance) depends on the instrument, and the microorganism and its metabolism. Generation times may be calculated based on TTD methods as described in Section 1.2.3.1, or from the time required for a doubling of the change in conductance.[37] Impedimetric instruments are often automated, allowing a large number of samples to run at the same time. Conductance has been used for modeling the growth of *Y. enterocolitica*[95] and impedance and conductance have been used for modeling the growth of *S. enteritidis*.[96,97] An indirect conductimetry method, in which CO_2 evolved during growth was trapped and measured, was proposed for the modeling of food spoilage by yeasts.[98]

1.3 CONCLUSION

It is important that a deliberate choice be made when choosing an experimental design or a method of data collection. The outcome of an experiment, and the ultimate value of the model, will be greatly influenced by the experimenter's choices. Selection of a data collection method involves some trade-off. The novel methods described above can roughly be divided into two groups, one that provides a possibility of automation and thereby allows a higher number of experiments to be analyzed, and another that gives additional information, e.g., on the physiological state of the microorganisms compared with viable counts. Turbidity and impedimetric methods are mainly in the first group, and flow cytometry and microscopy in the second. Although the viable count method probably remains the method of reference and of choice, it does not always give the correct answer, which was also pointed out in Section 1.2.3.2. It is expected that novel techniques for data collection will continue to increase in importance with the demand for more mechanistic models based on microbial physiology.

REFERENCES

1. Baranyi, J., Ross, T., McMeekin, T.A., and Roberts, T.A., Effects of parameterization on the performance of empirical models used in "predictive microbiology," *Food Microbiol.,* 13, 83, 1996.

2. Pin, C., Baranyi, J., and de Fernando, G., Predictive model for the growth of *Yersinia enterocolitica* under modified atmospheres, *J. Appl. Microbiol.,* 88, 521, 2000.
3. Masana, M.O. and Baranyi, J., Adding new factors to predictive models: the effect on the risk of extrapolation, *Food Microbiol.,* 17, 367, 2000.
4. Chhabra, A.T., Carter, W.H., Linton, R.H., and Cousin, M.A., A predictive model to determine the effects of pH, milk fat, and temperature on thermal inactivation of *Listeria monocytogenes, J. Food Prot.*, 62, 1143, 1999.
5. Uljas, H.E., Schaffner, D.W, Duffy, S., Zhao, L.H., and Ingham, S.C., Modeling of combined processing steps for reducing *Escherichia coli* O157:H7 populations in apple cider, *Appl. Environ. Microbiol.*, 67, 133, 2001.
6. Zhao, L., Montville, T.J., and Schaffner, D.W., Inoculum size of *Clostridium botulinum* 56A spores influences time-to-detection and percent growth-positive samples. *J. Food Sci.,* 65, 1369, 2000.
7. Buchanan, R.L. and Bagi, L.K., Microbial competition: effect of *Pseudomonas fluorescens* on the growth of *Listeria monocytogenes, Food Microbiol.,* 16, 523, 1999.
8. Rasch, M., The influence of temperature, salt and pH on the inhibitory effect of reuterin on *Escherichia coli, Int. J. Food Microbiol.,* 72, 225, 2002.
9. Membre, J.M., Ross, T., and McMeekin, T., Behaviour of *Listeria monocytogenes* under combined chilling processes, *Lett. Appl. Microbiol.,* 28, 216, 1999.
10. Farber, J.M., McKellar, R.C., and Ross, W.H., Modelling the effects of various parameters on the growth of *Listeria monocytogenes* on liver pate, *Food Microbiol.,* 12, 447, 1995.
11. Nerbrink, E., Borch, E., Blom, H., and Nesbakken, T., A model based on absorbance data on the growth rate of *Listeria monocytogenes* and including the effects of pH, NaCl, Na-lactate and Na-acetate, *Int. J. Food Microbiol.,* 47, 99, 1999.
12. Juneja, V.K. and Eblen, B.S., Predictive thermal inactivation model for *Listeria monocytogenes* with temperature, pH, NaCl, and sodium pyrophosphate as controlling factors, *J. Food Prot.,* 62, 986, 1999.
13. Juneja, V.K., Marmer, B.S., and Eblen, B.S., Predictive model for the combined effect of temperature, pH, sodium chloride, and sodium pyrophosphate on the heat resistance of *Escherichia coli* O157:H7, *J. Food Saf.,* 19, 147, 1999.
14. Khuri, A.I. and Cornell, J.A., *Response Surfaces. Design and Analyses,* Marcel Dekker, New York, 1987.
15. Battey, A.S. and Schaffner, D.W., Modelling bacterial spoilage in cold-filled ready to drink beverages by *Acinetobacter calcoaceticus* and *Gluconobacter oxydans, J. Appl. Microbiol.,* 91, 237, 2001.
16. Battey, A.S., Duffy, S., and Schaffner, D.W., Modelling mould spoilage in cold-filled ready-to-drink beverages by *Aspergillus niger* and *Penicillium spinulosum, Food Microbiol.,* 18, 521, 2001.
17. Battey, A.S., Duffy, S., and Schaffner, D.W., Modeling yeast spoilage in cold-filled ready-to-drink beverages with *Saccharomyces cerevisiae, Zygosaccharomyces bailii,* and *Candida lipolytica, Appl. Environ. Microbiol.,* 68, 1901, 2002.
18. Devlieghere, F., Debevere, J., and Van Impe, J., Concentration of carbon dioxide in the water-phase as a parameter to model the effect of a modified atmosphere on microorganisms, *Int. J. Food Microbiol.,* 43, 105, 1998.
19. Walls, I. and Scott, V.N., Validation of predictive mathematical models describing the growth of *Listeria monocytogenes, J. Food Prot.,* 60, 1142, 1997.
20. Guerzoni, M.E., Vannini, L., Lanciotti, R., and Gardini, F., Optimisation of the formulation and of the technological process of egg-based products for the prevention of *Salmonella enteritidis* survival and growth, *Int. J. Food Microbiol.,* 73, 367, 2002.

21. Lebert, I., Bégot, C., and Lebert, A., Development of two *Listeria monocytogenes* growth models in a meat broth and their application to beef meat, *Food Microbiol.,* 15, 499, 1998.
22. Lebert, I., Robles-Olvera, V., and Lebert, A., Application of polynomial models to predict growth of mixed cultures of *Pseudomonas* spp. and *Listeria* in meat, *Int. J. Food Microbiol.,* 61, 27, 2000.
23. Cheroutre-Vialette, M. and Lebert, A., Growth of *Listeria monocytogenes* as a function of dynamic environment at 10°C and accuracy of growth predictions with available models, *Food Microbiol.,* 17, 83, 2000.
24. Cheroutre-Vialette, M. and Lebert, A., Modelling the growth of *Listeria monocytogenes* in dynamic conditions, *Int. J. Food Microbiol.,* 55, 201, 2000.
25. Quignon, F., Huyard, A., Schwartzbrod, L., and Thomas, F., Use of Doehlert matrices for study of poliovirus-1 adsorption, *J. Virol. Methods,* 68, 33, 1997.
26. Terebiznik, M.R., Jagus, R.J., Cerrutti, P., de Huergo, M.S., and Pilosof, A.M.R., Combined effect of nisin and pulsed electric fields on the inactivation of *Escherichia coli, J. Food Prot.,* 63, 741, 2000.
27. Terebiznik, M., Jagus, R.J., Cerrutti, P., de Huergo, M.S., and Pilosof, A.M.R., Inactivation of *Escherichia coli* by a combination of nisin, pulsed electric fields, and water activity reduction by sodium chloride, *J. Food Prot.,* 65, 1253, 2002.
28. Bouttefroy, A., Linder, M., and Milliere, J.B., Predictive models of the combined effects of curvaticin 13, NaCl and pH on the behaviour of *Listeria monocytogenes* ATCC 15313 in broth, *J. Appl. Microbiol.,* 88, 919, 2000.
29. Bouttefroy, A., Mansour, M., Linder, M., and Milliere, J.B., Inhibitory combinations of nisin, sodium chloride, and pH on *Listeria monocytogenes* ATCC 15313 in broth by an experimental design approach, *Int. J. Food Microbiol.,* 54, 109, 2000.
30. Sautour, M., Rouget, A., Dantigny, P., Divies, G., and Bensoussan, M., Application of Doehlert design to determine the combined effects of temperature, water activity and pH on conidial germination of *Penicillium chrysogenum, J. Appl. Microbiol.,* 91, 900, 2001.
31. Versyck, K.J., Bernaerts, K., Geeraerd, A.H., and Van Impe, J.F., Introducing optimal experimental design in predictive modeling: a motivating example, *Int. J. Food Microbiol.,* 51, 39, 1999.
32. Bernaerts, K., Versyck, K.J., and Van Impe, J.F., On the design of optimal dynamic experiments for parameter estimation of a Ratkowsky-type growth kinetics at suboptimal temperatures, *Int. J. Food Microbiol.,* 54, 27, 2000.
33. Bernaerts, K., Servaes, R.D., Kooyman, S., Versyck, K.J., and Van Impe, J.F., Optimal temperature input design for estimation of the square root model parameters: parameter accuracy and model validity restrictions, *Int. J. Food Microbiol.,* 73, 145, 2002.
34. Ratkowsky, D.A., Olley, J., McMeekin, T.A., and Ball, A., Relationship between temperature and growth rate of bacterial cultures, *J. Bacteriol.,* 149, 1, 1982.
35. Baranyi, J. and Roberts, T.A., A dynamic approach to predicting bacterial growth in food, *Int. J. Food Microbiol.,* 23, 277, 1994.
36. Grijspeerdt, K. and Vanrolleghem, P., Estimating the parameters of the Baranyi model for bacterial growth, *Food Microbiol.,* 16, 593, 1999.
37. McMeekin, T.A., Olley, J.N., Ross, T., and Ratkowsky, D., *Predictive Microbiology: Theory and Application,* John Wiley & Sons, New York, 1993, 340 pp .
38. Neumeyer, K., Ross, T., and McMeekin, T.A., Development of a predictive model to describe the effects of temperature and water activity on the growth of spoilage pseudomonads, *Int. J. Food Microbiol.,* 38, 45, 1997.

39. Neumeyer, K., Ross, T., Thomson, G., and McMeekin, T.A., Validation of a model describing the effects of temperature and water activity on the growth of psychrotrophic pseudomonads, *Int. J. Food Microbiol.,* 38, 55, 1997.
40. McElroy, D.M., Jaykus, L.A., and Foegeding, P.M., Validation and analysis of modeled predictions of growth of *Bacillus cereus* spores in boiled rice, *J. Food Prot.,* 63, 268, 2000.
41. Miles, D.W., Ross, T., Olley, J., and McMeekin, T.A., Development and evaluation of a predictive model for the effect of temperature and water activity on the growth rate of *Vibrio parahaemolyticus, Int. J. Food Microbiol.,* 38, 133, 1997.
42. Benito, A., Ventoura, G., Casadei, M., Robinson, T., and Mackey, B., Variation in resistance of natural isolates of *Escherichia coli* O157 to high hydrostatic pressure, mild heat, and other stresses, *Appl. Environ. Microbiol.,* 65, 1564, 1999.
43. Oscar, T.P., Development and validation of a tertiary simulation model for predicting the potential growth of *Salmonella typhimurium* on cooked chicken, *Int. J. Food Microbiol.,* 76, 177, 2002.
44. Hudson, J.A., Comparison of response surface models for *Listeria monocytogenes* strains under aerobic conditions, *Food Res. Int.,* 27, 53, 1994.
45. Begot, C., Lebert, I., and Lebert, A., Variability of the response of 66 *Listeria monocytogenes* and *Listeria innocua* strains to different growth conditions, *Food Microbiol.,* 14, 403, 1997.
46. Whiting, R.C. and Golden, M.H., Variation among *Escherichia coli* O157:H7 strains relative to their growth, survival, thermal inactivation, and toxin production in broth, *Int. J. Food Microbiol.,* 75, 127, 2002.
47. Oscar, T.P., Variation of lag time and specific growth rate among 11 strains of *Salmonella* inoculated onto sterile ground chicken breast burgers and incubated at 25°C, *J. Food Saf.,* 20, 225, 2000.
48. Salter, M.A., Ross, T., and McMeekin, T.A., Applicability of a model for nonpathogenic *Escherichia coli* for predicting the growth of pathogenic *Escherichia coli, J. Appl. Microbiol.,* 85, 357, 1998.
49. McClure, P.J., Blackburn, C.W., Cole, M.B., Curtis, P.S., Jones, J.E., Legan, J.D., Ogden, I.D., Peck, M.W., Roberts, T.A., Sutherland, J.P., and Walker, S.J., Modelling the growth, survival and death of microorganisms in foods: the UK Food Micromodel approach, *Int. J. Food Microbiol.,* 23, 265, 1994.
50. Stewart, C.M., Cole, M.B., Legan, J.D., Slade, L., Vandeven, M.H., and Schaffner, D.W., Modeling the growth boundary of *Staphylococcus aureus* for risk assessment purposes, *J. Food Prot.,* 64, 51, 2001.
51. Rattanasomboon, N., Bellara, S.R., Harding, C.L., Fryer, P.J., Thomas, C.R., Al-Rubeai, M., and McFarlane, C.M., Growth and enumeration of the meat spoilage bacterium *Brochothrix thermosphacta, Int. J. Food Microbiol.,* 51, 145, 1999.
52. Gardini, F., Lanciotti, R., Sinigaglia, M., and Guerzoni, M.E., A head space gas chromatographic approach for the monitoring of the microbial cell activity and the cell viability evaluation, *J. Microbiol. Methods,* 29, 103, 1997.
53. Guerzoni, M.E., Lanciotti, R., Torriani, S., and Dellaglio, F., Growth modeling of *Listeria monocytogenes* and *Yersinia enterocolitica* in food model systems and dairy products, *Int. J. Food Microbiol.,* 24, 83, 1994.
54. Ellison, A., Anderson, W., Cole, M.B., and Stewart, G.S.A.B., Modeling the thermal inactivation of *Salmonella typhimurium* using bioluminescence data, *Int. J. Food Microbiol.,* 23, 467, 1994.

55. Farkas, J., Andrassy, E., Beczner, J., Vidacs, I., and Meszaros, L., Utilizing luminometry for monitoring growth of *Listeria monocytogenes* in its liquid or gelified monocultures and cocultures with "acid-only" *Lactococcus lactis, Int. J. Food Microbiol.,* 73, 159, 2002.
56. Dalgaard, P., Ross, T., Kamperman, L., Neumeyer, K., and McMeekin, T.A., Estimation of bacterial growth rates from turbidimetric and viable count data, *Int. J. Food Microbiol.,* 23, 391, 1994.
57. McClure, P.J., Cole, M.B., Davies, K.W., and Anderson, W.A., The use of turbidimetric data for the construction of kinetic models, *J. Ind. Microbiol.,* 12, 277, 1993.
58. Hudson, J.A. and Mott, S.J., Comparison of lag times obtained from optical-density and viable count data for a strain of *Pseudomonas fragi, J. Food Saf.,* 14, 329, 1994.
59. Huchet, V., Thuault, D., and Bourgeois, C.M., Development of a model predicting the effects of pH, lactic acid, glycerol and sodium chloride content on the growth of vegetative cells of *Clostridium tyrobutyricum* in a culture medium, *Lait*, 75, 585, 1995.
60. McKellar, R.C. and Knight, K., A combined discrete–continuous model describing the lag phase of *Listeria monocytogenes, Int. J. Food Microbiol.,* 54, 171, 2000.
61. Augustin, J.C., Rosso, L., and Carlier, V., Estimation of temperature dependent growth rate and lag time of *Listeria monocytogenes* by optical density measurements, *J. Microbiol. Methods,* 38, 137, 1999.
62. Chorin, E., Thuault, D., Cleret, J.J., and Bourgeois, C.M., Modelling *Bacillus cereus* growth, *Int. J. Food Microbiol.,* 38, 229, 1997.
63. Breand, S., Fardel, G., Flandrois, J.P., Rosso, L., and Tomassone, R., A model describing the relationship between lag time and mild temperature increase duration, *Int. J. Food Microbiol.,* 38, 157, 1997.
64. Cuppers, H.G.A.M. and Smelt, J.P.P.M., Time to turbidity measurement as a tool for modeling spoilage by *Lactobacillus, J. Ind. Microbiol.,* 12, 168, 1993.
65. Baranyi, J. and Pin, C., Estimating bacterial growth parameters by means of detection times, *Appl. Environ. Microbiol.,* 65, 732, 1999.
66. Jenkins, P., Poulos, P.G., Cole, M.B., Vandeven, M.H., and Legan, J.D., The boundary for growth of *Zygosaccharomyces bailii* in acidified products described by models for time to growth and probability of growth, *J. Food Prot.,* 63, 222, 2000.
67. Masana, M.O. and Baranyi, J., Growth/no growth interface of *Brochothrix thermosphacta* as a function of pH and water activity, *Food Microbiol.,* 17, 485, 2000.
68. McKellar, R.C., Lu, X., and Delaquis, P.J., A probability model describing the interface between survival and death of *Escherichia coli* O157:H7 in a mayonnaise model system, *Food Microbiol.,* 19, 235, 2003.
69. Vives-Rego, J., Lebaron, P., and Nebe-von Caron, G., Current and future applications of flow cytometry in aquatic microbiology, *FEMS Microbiol. Rev.,* 24, 429, 2000.
70. Walberg, M., Gaustad, P., and Steen, H.B., Rapid assessment of ceftazidime, ciprofloxacin, and gentamicin susceptibility in exponentially-growing *E. coli* cells by means of flow cytometry, *Cytometry,* 27, 169, 1997.
71. Smelt, J.P.P.M., Otten, G.D., and Bos, A.P., Modelling the effect of sublethal injury on the distribution of the lag times of individual cells of *Lactobacillus plantarum, Int. J. Food Microbiol.,* 73, 207, 2002.
72. Jacobsen, C.N., Rasmussen, J., and Jakobsen, M., Viability staining and flow cytometric detection of *Listeria monocytogenes, J. Microbiol. Methods,* 28, 35, 1997.
73. Sørensen, B.B. and Jakobsen, M., The combined effects of temperature, pH and NaCl on growth of *Debaryomyces hansenii* analyzed by flow cytometry and predictive microbiology, *Int. J. Food Microbiol.,* 34, 209, 1997.

74. Dalgaard, P. and Koutsoumanis, K., Comparison of maximum specific growth rates and lag times estimated from absorbance and viable count data by different mathematical models, *J. Microbiol. Methods,* 43, 183, 2001.
75. Budde, B.B., Siegumfeldt, H., and Rasch, M., The potential use of flow cytometry in predictive microbiology, in *COST 914: Predictive Modelling of Microbial Growth and Survival in Foods*, Roberts, T.A., Ed., Office for Official Publications of the European Communities, Luxembourg, 1999, p. 271.
76. Ueckert, J., Nebe-von Caron, G., Bos, A.P., and ter Steeg, P.F., Flow cytometric analysis of *Lactobacillus plantarum* to monitor lag times, cell division and injury, *Lett. Appl. Microbiol.,* 25, 295, 1997.
77. Nebe-von Caron, G., Stephens, P., and Badley, R.A., Assessment of bacterial viability status by flow cytometry and single cell sorting, *J. Appl. Microbiol.,* 84, 988, 1998.
78. Budde, B.B. and Rasch, M., A comparative study on the use of flow cytometry and colony forming units for assessment of the antibacterial effect of bacteriocins, *Int. J. Food Microbiol.,* 63, 65, 2001.
79. García-Ochoa, F., Santos, V.E., and Alcon, A., Intracellular compounds quantification by means of flow cytometry in bacteria: application to xanthan production by *Xanthomonas campestris, Biotech. Bioeng.,* 57, 87, 1998.
80. de Alteriis, E., Porro, D., Romano, V., and Parascandola, P., Relation between growth dynamics and diffusional limitations in *Saccharomyces cerevisiae* cells growing as entrapped in an insolubilised gelatin gel, *FEMS Microbiol. Lett.,* 195, 245, 2001.
81. Guldfeldt, L.U. and Arneborg, N., Measurement of the effects of acetic acid and extracellular pH on intracellular pH of nonfermenting, individual *Saccharomyces cerevisiae* cells by fluorescence microscopy, *Appl. Environ. Microbiol.,* 64, 530, 1998.
82. Shabala, L., Budde, B., Ross, T., Siegumfeldt, H., Jakobsen, M., and McMeekin, T., Responses of *Listeria monocytogenes* to acid stress and glucose availability revealed by a novel combination of fluorescence microscopy and microelectrode ion-selective techniques, *Appl. Environ. Microbiol.,* 68, 1794, 2002.
83. Wu, Y., Griffiths, M.W., and McKellar, R.C., A comparison of the Bioscreen method and microscopy for the determination of lag times of individual cells of *Listeria monocytogenes, Lett. Appl. Microbiol.,* 30, 468, 2000.
84. Wright, K.M., Coleman, H.P., Mackle, A.R., Parker, M.L., Brocklehurst, T.F., Wilson, D.R., and Hills, B.P., Determination of mean growth parameters of bacterial colonies immobilized in gelatin gel using a laser gel-cassette scanner, *Int. J. Food Microbiol.,* 57, 75, 2000.
85. Harrison, W.A., Peters, A.C., and Fielding, L.M., Growth of *Listeria monocytogenes* and *Yersinia enterocolitica* colonies under modified atmospheres at 4 and 8°C using a model food system, *J. Appl. Microbiol.,* 88, 38, 2000.
86. Dykes, G.A., Image analysis of colony size for investigating sublethal injury in *Listeria monocytogenes, J. Rapid Methods Autom. Microbiol.,* 7, 223, 1999.
87. Stecchini, M.L., Del Torre, M., Donda, S., and Maltini, E., Growth of *Bacillus cereus* on solid media as affected by agar, sodium chloride, and potassium sorbate, *J. Food Prot.,* 63, 926, 2000.
88. Salvesen, I. and Vadstein, O., Evaluation of plate count methods for determination of maximum specific growth rate in mixed microbial communities, and its possible application for diversity assessment, *J. Appl. Microbiol.,* 88, 442, 2000.
89. Gibson, A.M., Baranyi, J., Pitt, J.I., Eyles, M.J., and Roberts, T.A., Predicting fungal growth: the effect of water activity on *Aspergillus flavus* and related species, *Int. J. Food Microbiol.,* 23, 419, 1994.

90. Valík, L., Baranyi, J., and Gorner, F., Predicting fungal growth: the effect of water activity on *Penicillium roqueforti, Int. J. Food Microbiol.,* 47, 141, 1999.
91. Baranyi, J., Roberts, T.A., and McClure, P., A non-autonomous differential equation to model bacterial growth, *Food Microbiol.,* 10, 43, 1993.
92. Valík, L. and Piecková, E., Growth modelling of heat-resistant fungi: the effect of water activity, *Int. J. Food Microbiol.,* 63, 11, 2001.
93. Rosso, L. and Robinson, T.P., A cardinal model to describe the effect of water activity on the growth of moulds, *Int. J. Food Microbiol.,* 63, 265, 2001.
94. Firstenberg-Eden, R. and Eden, G., *Impedance Microbiology,* John Wiley & Sons, New York, 1984, 170 pp .
95. Lindberg, C.W. and Borch, E., Predicting the aerobic growth of *Yersinia enterocolitica* O-3 at different pH-values, temperatures and L-lactate concentrations using conductance measurements, *Int. J. Food Microbiol.,* 22, 141, 1994.
96. Fehlhaber, K. and Kruger, G., The study of *Salmonella enteritidis* growth kinetics using rapid automated bacterial impedance technique, *J. Appl. Microbiol.,* 84, 945, 1998.
97. Koutsoumanis, K., Tassou, C.C., Taoukis, P.S., and Nychas, G.J.E., Modelling the effectiveness of a natural antimicrobial on *Salmonella enteritidis* as a function of concentration, temperature and pH, using conductance measurements, *J. Appl. Microbiol.,* 84, 981, 1998.
98. Deak, T. and Beuchat, L.R., Use of indirect conductimetry for predicting growth of food spoilage yeasts under various environmental conditions, *J. Ind. Microbiol.,* 12, 301, 1993.